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## Bacterial Endotoxin: Molecular Relationships Between Structure and Activity

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Fever, granulocytopenia followed by granulocytosis, and decreases in platelet counts and blood pressure are general symptoms of systemic infections caused by gram-negative bacteria such as *Escherichia*, *Pseudomonas*, and *Neisseria*.<sup>95</sup> In severe cases, the infection may lead to shock and death. These effects of gram-negative pathogens are independent of bacterial viability because they can also be elicited by the injection of killed cells. One active principle that participates in the pathogenic potential of gram-negative bacteria and the induction of toxic reactions resides in the bacterial cell envelope. It has been termed *endotoxin* because of its close association with the cell wall (the exotoxins, in contrast, are actively secreted by viable bacteria into the environment).<sup>50, 93</sup>

There is ample evidence that endotoxins figure prominently in the pathogenicity of wild-type strains of bacteria. Endotoxin seems to contribute to the resistance of pathogenic bacteria against the bactericidal action of serum and the intracellular killing by phagocytes.<sup>26, 45, 52</sup> In addition, it is likely that endotoxin released from bacteria during infection represents the causative agent of certain manifestations of infection and sepsis. This hypothesis is attractive in view of the remarkable similarity between symptoms of gram-negative bacteremia and the biologic effects of injected endotoxin, and it is supported by the fact that multiplying microbes do actually release endotoxin.<sup>13</sup>

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the role of endotoxin during bacterial sepsis.<sup>34, 35, 36</sup> concomitantly with the improvement of the sensitivity and specificity of the limulus amebocyte lysate test that is used to estimate endotoxin levels in body fluids and tissues,<sup>42</sup> more recent studies clearly support the significance of endotoxin in septicemic states. Thus, in several investigations a positive limulus test in human blood or plasma correlated with hypotension and increased mortality<sup>43</sup>; for further studies see references 87 and 91.

In fact, endotoxemia proved to be a reliable predictor of gram-negative septicemia.<sup>10</sup> A recent study<sup>10</sup> reports that in seven out of eight patients with fulminant meningococcal septicemia, endotoxin plasma levels exceeded 800 pg/ml. Seven of these patients developed adult respiratory distress syndrome (ARDS) and renal dysfunction, and four died. In eight patients with less severe meningitis, endotoxin levels were lower (85 to 260 pg/ml) or absent (five patients). None of these patients developed multi-organ failure or septic shock. In plasma of a third group of patients with positive blood cultures but no signs of circulatory, respiratory, or renal abnormalities, endotoxin could not be detected in plasma or was present in only low concentrations. This study therefore demonstrates a quantitative relationship between high endotoxin levels in plasma and the development of septic shock and multiple organ failure. Although in this study the threshold dose of endotoxin causing deleterious effects was on the order of 800 pg/ml, it is possible that in certain clinical settings even substantially lower endotoxin concentrations, likely to escape detection with the limulus test, may be relevant.

Moreover, experimental animals and humans become up to 1000-fold more sensitive to endotoxin during gram-negative bacterial infection.<sup>19</sup> The mechanism of this sensitization is not known, but it may relate to the phenomenon that ubiquitous bacterial cell wall components including muramyl dipeptide (MDP), outer membrane proteins, and endotoxin itself can greatly sensitize animals to lethal endotoxicity.<sup>24, 34</sup> In addition, we know that products from gram-positive bacteria (such as MDP, the staphylococcal toxic shock syndrome toxin 1, or the pyrogenic streptococcal exotoxin) dramatically increase the sensitivity of rabbits to the lethal action of endotoxin.<sup>12, 36</sup> Thus, it appears possible that during infection bacterial components also greatly increase host susceptibility to endotoxin, thereby rendering otherwise harmless endotoxin concentrations highly toxic.

The significance of endotoxins in bacterial infection and their role as bacterial surface antigens (O antigens) have stimulated investigations into their chemical nature and the mechanisms of their biologic action during the last few decades. These studies have generated an overwhelming amount of data and number of concepts. The progress made in these fields has been summarized in the *Handbook of Endotoxin*<sup>60</sup> as well as in recent proceedings of endotoxin conferences.<sup>17, 59, 97, 98</sup> This article summarizes some recent results regarding the chemical and physical structure of the biologically active domains of endotoxins and emphasizes structure-activity relationships. Of the enormous literature available, only recent reviews and selected newer original publications are cited.

Endotoxins of various gram-negative bacterial families consist of a polysaccharide portion and a covalently bound lipid component, called lipid A, and hence, they are chemically lipopolysaccharides (Fig. 1).<sup>50</sup> The polysaccharide component of enterobacterial lipopolysaccharides (LPSs) consists of two regions that differ in their genetic determination, biosynthesis, and architecture. These regions are the O-specific chain and the core oligosaccharide (see Fig. 1). A variety of nonenterobacterial wild-type strains of phototrophic and some human pathogenic gram-negative bacteria including *Neisseria*, *Acinetobacter*, *Bordetella*, *Bacteroides*, and *Haemophilus* form lipopolysaccharides that contain only an oligosaccharide and the lipid A component but lack the typical O-specific chain.<sup>25, 31</sup>

#### O-Specific Chain

The O-specific chain is a polymer of repeating oligosaccharide units that contain up to six sugar residues. A large diversity of the constituent glycosyl residues of repeating units has been revealed within different gram-negative bacteria. The nature, ring form, type of linkage, and type of substitution of the individual monosaccharide residues as well as their sequence within a repeating unit is characteristic and unique for a given lipopolysaccharide and the parental bacterial strain, i.e., a bacterial species.<sup>50</sup> As an example, Figure 2 shows the structure of the repeating unit of the O-specific chain of two *Salmonella* species, *S. abortus equi* and *S. typhi*. In both cases a backbone trisaccharide of D-mannose (Man), L-rihamnose (Rha), and D-galactose (Gal) is present, which is substituted by D-glucose (Glc) and a 3,6-dideoxy sugar in the linkages depicted. In *S. abortus equi* abequose (Abe, 3,6-dideoxy-D-galactose) and in *S. typhi* tyvelose (Twy, 3,6-dideoxy-D-mannose) are present and differ only in the orientation of two hydroxyl groups.

Because of the abundance and diversity of theoretically possible constituents and their linkages, an enormous number of structures of O-specific chains is conceivable; this is verified by nature. Thus, an immense structural variability is revealed when the O-specific chains of distinct bacterial origin are compared.

#### Core Oligosaccharide

The core region of enterobacterial lipopolysaccharides consists of a hetero-oligosaccharide, which can be formally subdivided into the O-chain-proximal outer core and the lipid A proximal inner core. The outer core contains the common sugars D-glucose (Glc), D-galactose (Gal), N-acetyl-D-glucosamine (GlcNAc), and N-acetyl-D-galactosamine (GalNAc), whereas the inner core region is composed of the uncommon sugars heptose, mainly in the *L-glycero-D-manno* (*L*,*D*-Hep) and the *D-glycero-D-manno* config-

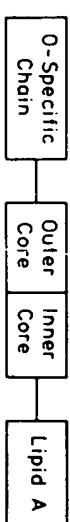


Figure 1. Schematic architecture of an enterobacterial lipopolysaccharide. (From Rietzschel E-Th, Bräde L, Schade UJ, et al: Bacterial endotoxins: Properties and structure of biologically active domains. In Schäffer E, Richmond M, Seibert G (eds): Surface Structures of Microorganisms and their Interaction with the Mammalian Host. Weinheim, Germany, Verlag Chemie, 1988, pp 5-13, with permission.)

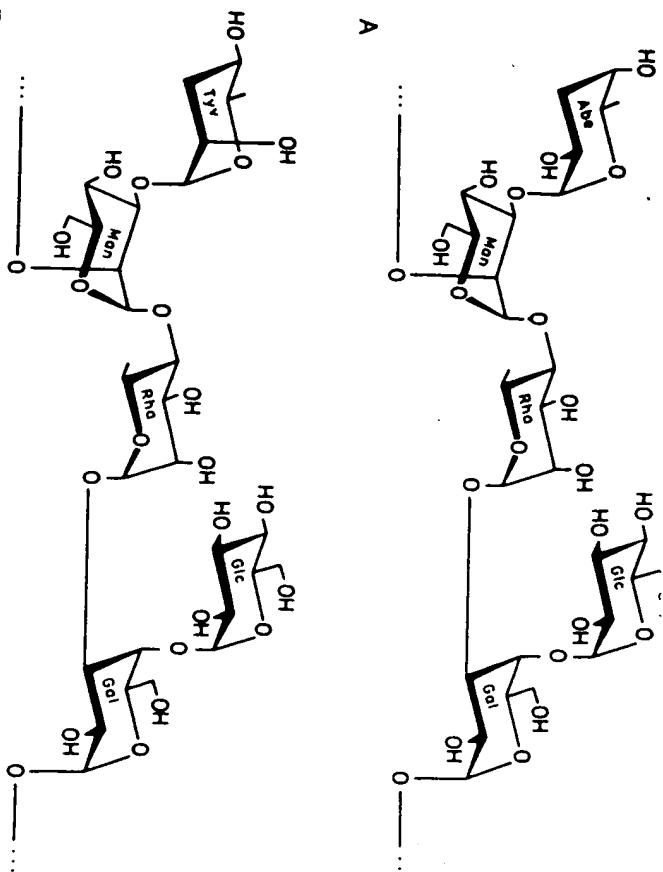


Figure 2. Chemical structure of the repeating unit in lipopolysaccharide of *Salmonella abortus equi* (A) and *Salmonella typhi* (B). Compare with references 50 and 52.

different bacterial species is limited.<sup>50, 55</sup> For example, in the genus *Salmonella* only one core type (Ra core) occurs in all serotypes; in *Escherichia coli*, so far five core types (R1, R2, R3, R4, and K-12) have been identified; in *Proteus* also five core types have been found; and in *Citrobacter* four core types have been described for the many serotypes within these genera.<sup>55</sup> The moderate variability of core types occurs primarily in the outer region, while the Kdo-containing inner core appears to be naturally even more conserved.

We have analyzed the structure of the Kdo-containing inner core of *Salmonella* lipopolysaccharides using the lipopolysaccharide of the *S. minnesota* Rd<sub>1</sub>P<sub>1</sub> mutant (strain R7), which lacks the O-specific chain and the outer core.<sup>4, 53</sup> Taking previous studies<sup>4, 50</sup> into account, the carbohydrate structure of the inner core region of *Salmonella* can be described as shown in Figure 3. Accordingly, one Kdo residue is present in the main core oligosaccharide chain (Kdo I). It is substituted in position 4 by an  $\alpha$ -linked Kdo II, which in turn may carry, at position 4, nonstoichiometric amounts of a further  $\alpha$ -linked Kdo residue (Kdo III). All Kdo groups are present as pyranosides. In its position 5, the Kdo I residue carries  $\alpha$ -bound L,D-Hep (Hep I) to which a second L,D-Hep residue (Hep II) is  $\alpha$ -linked (position 3). The latter is substituted in position 7 by a third L,D-Hep group (Hep

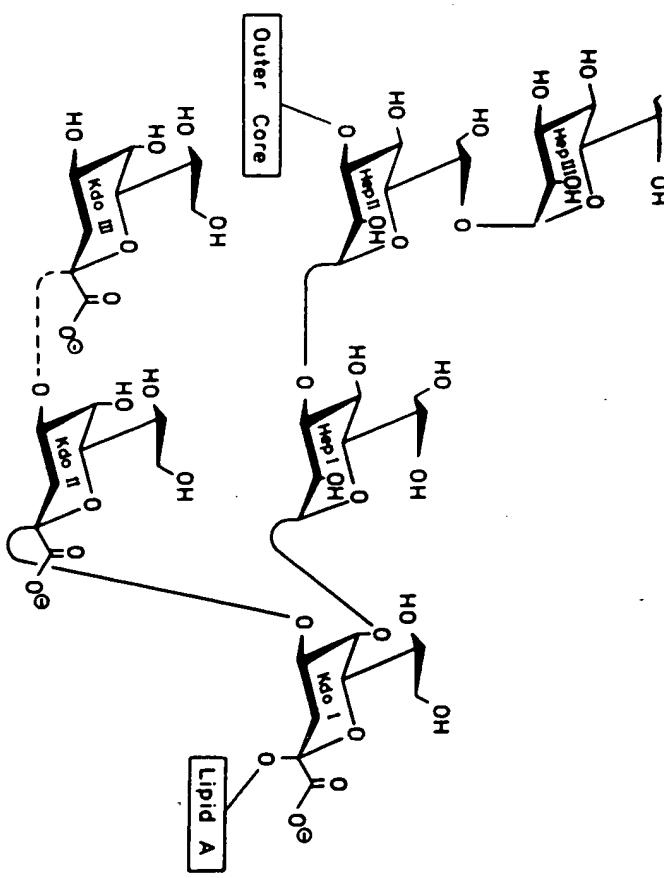


Figure 3. Chemical structure of the inner core region of *Salmonella minnesota* lipopolysaccharide. Phosphate, pyrophosphate, and 2-aminoethyl(pyrophosphate), which are linked to the inner core, are not shown. Kdo III is not always present in stoichiometric amounts as indicated by the dashed line. (From Rietschel ET, Bräde L, Schade UF, et al: Bacterial endotoxins: Properties and structure of biologically active domains. In Schrinner E, Richmond N, Seibert G (eds): Surface Structures of Microorganisms and their Interaction with the Mammalian Host. Weinheim, Germany: Verlag Chemie, 1988, pp 5-13; with permission.)

III). In lipopolysaccharides of less defective mutants and wild-type bacteria, the saccharide chain extends from the hydroxyl group in position 3 of Hep II (see Fig. 3). Kdo I is  $\alpha$ -ketosidically linked to the primary hydroxyl group (position 6') of the nonreducing (distal) D-glucosamine residue (GlcN II) of the lipid A backbone (Figs. 4 and 5).

The structure shown in Figure 3 does not take into account that the inner core region is substituted at various sites by phosphate, pyrophosphate, 2-aminoethylphosphate, or 2-aminoethylpyrophosphate. In addition, in *E. coli* lipopolysaccharide possessing the R3 core type, GlcN may be attached to L,D-Hep.<sup>53</sup> Kdo, on the other hand, may carry a great variety of different substituents. Depending on the bacterial origin of lipopolysaccharide, the hydroxyl group in position 4 is substituted in general by negatively charged groups [e.g., Kdo, phosphate, D-galacturonic, and D-glucuronic acid], position 5 by neutral sugars [Hep, Man, Rha], position 7 by Gal and 2-aminoethyl phosphate, and position 8 by either positively or negatively charged residues (e.g., phosphate, Kdo, or L-Ara4N).<sup>54, 55</sup> These inner core substituents are often not present in stoichiometric amounts, which is a further basis for the genuine lipopolysaccharide heterogeneity.

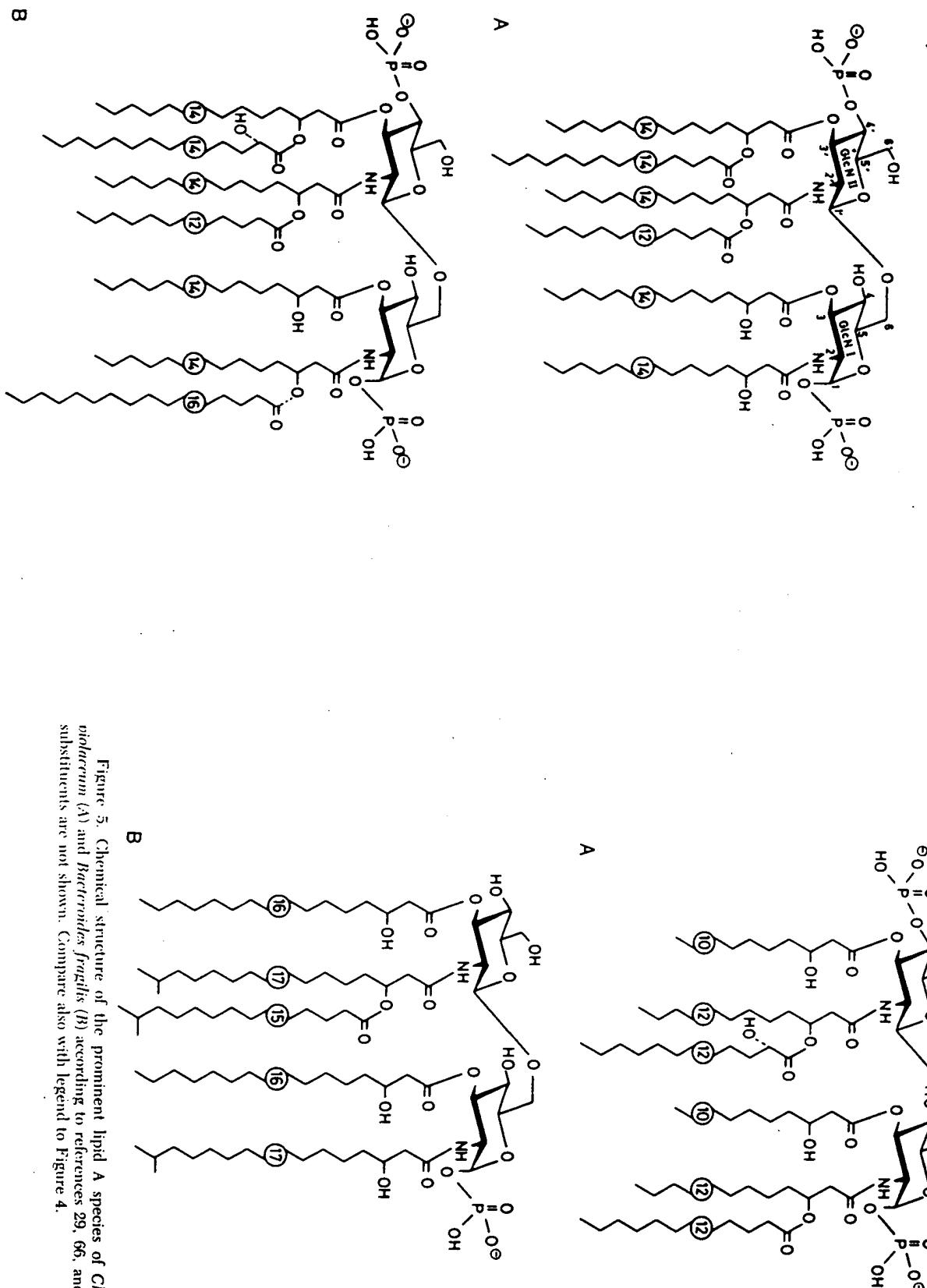


Figure 4. Chemical structure of the lipid A component of *Escherichia coli* (A) and *Salmonella minnesota* (B) according to reference 69. Substituents of phosphate groups are not shown (compare with text). The hydroxyl group in position 6 represents the attachment site of Kdo. The dotted line indicates nonstoichiometric substitution. Numbers in circles refer to the number of carbon atoms in acyl chains.

Despite this heterogeneity, lipopolysaccharides of Enterobacteriaceae and the majority of other bacteria studied (including those lacking the O-specific chain) contain Hep whereas *all* lipopolysaccharides, independent of their bacterial origin, harbor at least one  $\alpha$ -bound pyranosidic or furanosidic Kdo residue (or a derivative thereof) with a free carboxyl group that occupies the lipid A-proximal position of the reducing terminus of the inner core region. Therefore, Kdo or a derivative is a common and obligatory

Figure 5. Chemical structure of the prominent lipid A species of *Chromobacterium violaceum* (A) and *Bacteroides fragilis* (B) according to references 29, 66, and 78. Phosphate substituents are not shown. Compare also with legend to Figure 4.

region appears to be rather conserved.

### Lipid A

Lipid A represents the covalently linked lipid component of lipopolysaccharide.<sup>55, 65</sup> Enzymes that can cleave the polysaccharide-lipid bond are not known, and hence, polysaccharide-deprived free lipid A can be prepared only by chemical degradation, e.g., by acid-catalyzed hydrolysis of lipopolysaccharide. The ketosidic linkage between Kdo and lipid A is particularly acid-labile, and therefore, free lipid A is released on treatment of lipopolysaccharide with mild acid. Nevertheless, even the application of mild acid may cause some degradation and a certain heterogeneity of free lipid A. Lipid A also exhibits intrinsic heterogeneity, which is due to incomplete biosynthesis.<sup>64</sup>

Despite this, the primary structure of enterobacterial and some nonenterobacterial lipid A's has been elucidated in great detail. These structures were, in general, analyzed by using R-form lipopolysaccharide. In Figure 3, the primary structure of the predominant components of *Escherichia coli* (Fig. 4A) and *Salmonella minnesota* (Fig. 4B) lipid A is shown.<sup>40, 62</sup> In both cases, lipid A is composed of a  $\beta$ -D-glucosaminyl-(1-6)- $\alpha$ -D-glucosamine disaccharide that carries two phosphoryl groups, one in position 4' (of the distal glucosaminyl residue, GlcN II) and one in position 1 (of the reducing glucosaminyl residue, GlcN I). This hydrophilic lipid A backbone is, in both cases, acylated by four (primary) acyl groups, i.e., four (R)-3-hydroxymyristic acid residues [14:0(3-OH)] at positions 2, 3, 2', and 3'. As a further common feature, both lipid A's contain two unsubstituted hydroxyl groups at positions 4 and 6'. The latter primary hydroxyl group is free only in polysaccharide-deprived lipid A (termed free lipid A), for in lipopolysaccharide it represents the attachment site of Kdo, i.e., of the polysaccharide component.<sup>55</sup>

In both *E. coli* and *S. minnesota* lipid A, the hydroxyl groups of the two 14:0(3-OH) residues bound to GlcN II (positions 2' and 3') carry, as secondary acyl groups lauric (12:0) and myristic acid (14:0), respectively. In *S. minnesota* this myristoyl group is partially replaced by (S)-2-hydroxymyristic acid yielding two lipid A species. In *E. coli*, the two 14:0(3-OH) residues at GlcN I are not 3-O-acylated whereas in *S. minnesota* the amide-linked 14:0(3-OH) of GlcN I is either not acylated or carries palmitic acid (16:0). Two molecular species are therefore recognized that differ in the substitution of 14:0(3-OH) at GlcN I and, thus, in the number of acyl groups present. Obviously, one of these species structurally corresponds to hexaacyl *E. coli* lipid A.

In addition to the main structures shown in Figure 4, these lipid A preparations also contain molecules that possess the phosphorylated glucose-amine backbone but a smaller number of acyl groups. Such acyl-deficient structures are found particularly in enterobacterial S-form lipopolysaccharide.<sup>15, 34</sup> The phosphoryl residues in positions 1 and 4' may be substituted and phosphate (*E. coli*), 2-aminoethyl(pyrophosphate and 4-amino-4-deoxy-L-arabinopyranose (*L-Ara4N*, *S. minnesota*) have been identified as polar headgroups (not shown in Fig. 4). They are present in nonstoichiometric

amounts, which represents another facet of the intrinsic heterogeneity of lipid A.

The same structure shown for *E. coli* lipid A has been identified in *S. typhimurium*,<sup>55</sup> and this type of lipid A is also present in other enterobacterial and nonenterobacterial genera such as *Haemophilus*<sup>30</sup> and *Providencia*.<sup>53</sup> Both hexaacyl *E. coli* lipid A and heptaacyl *S. minnesota* lipid A have been chemically synthesized and shown to be identical in all chemical and physical properties to their purified bacterial counterpart (see following text).

Lipid A's of nonenterobacterial lipopolysaccharides differ in structure from those of *E. coli* and *S. minnesota* in several parameters, including the nature, number, chain length and location of fatty acids, and the absence of the nonglycosidic phosphate group at GlcN II. To illustrate such variations Figure 5 shows the chemical structure of the major components of lipid A of *Chromobacterium violaceum*<sup>29</sup> and *Bacteroides fragilis*.<sup>82</sup> In *E. coli* hexaacyl lipid A, GlcN II carries four and GlcN I two acyl groups. Thus, the acylation pattern over the two GlcN residues is *asymmetric* (4 + 2). In contrast, a *symmetric* (3 + 3) fatty acid distribution is recognized in hexaacyl *C. violaceum* lipid A (see Fig. 5A). Here, GlcN I and GlcN II each carry three fatty acids. (It should be mentioned that the amide-linked 12:0(3-OH) of GlcN II is 3-O-acylated by either 12:0 or (S)-2-hydroxydecanoic acid [12:0(2-OH)], yielding two molecular species.) Lipid A of *C. violaceum* further differs from *E. coli* in the chain length of its fatty acids. This type of lipid A has also been identified in *Neisseria gonorrhoeae*.<sup>85</sup> In *E. coli* lipid A, six fatty acids are present; however, in the major species of *B. fragilis* lipid A (see Fig. 5B) only a total of five fatty acids is detected because only one secondary acyl residue, bound to the 3-hydroxy fatty acid at position 2' at GlcN II, is present. The comparatively long-chain acyl groups of *B. fragilis* are, in part, isobranched and clearly different from those present in *E. coli* lipid A. The most striking difference to *E. coli* and the other lipid A's, however, is that in *B. fragilis* lipid A, the hydroxyl group at position 4' of GlcN II is free, i.e., the nonglycosidic phosphate group is lacking. It is noteworthy that *B. fragilis* LPS and lipid A have little endotoxic activity.<sup>43</sup>

In other lipid A's, additional compositional and architectural differences as compared to the *E. coli* prototype structure have been revealed. To name only a few examples, in endotoxically active *Campylobacter jejuni* lipid A, 2,3-diamino-2,3-dideoxy-D-glucose (GlcN3N) positionally replaces GlcN II of the backbone disaccharide<sup>55</sup>; in the nontoxic lipid A of *Rhodopseudomonas viridis*, an acylated GlcN3N-monosaccharide forms the lipid A backbone<sup>71</sup>; in *Rhodobacter sphaeroides*, 3-hydroxy fatty acids are, in part, replaced by 3-oxo fatty acids<sup>55, 85</sup>; and in *Rhizobium trifoli*, 27-hydroxyoctacosanoic acid, which is bound to D-glucosaminuronic acid,<sup>32</sup> represents the major lipid A acyl group (for further structural variations see references 4, 50, 53, 69, 70). Thus, lipid A's of distinct bacterial origin share certain basic structural elements, but they may differ in some other chemical features. Nevertheless, these analyses show that endotoxically active lipid A's of different bacterial origin are closely related in structure. Characteristic and common to them is the presence of a bisphosphorylated

ture has so far not been identified in other natural compounds, and hence, it is unique to lipid A.<sup>48</sup> The lipid A backbone carries, in general, approximately four mole equivalents of (R)-3-hydroxy fatty acids, two of which occupy amino functions and two of which are linked to the backbone hydroxyl groups at positions 3 and 3'. Both amide- and ester-bound (R)-3-hydroxy fatty acids are, in part, substituted at their 3-hydroxyl groups by saturated fatty acids. The resulting (R)-3-acyloxyacyl residues were found in evolutionarily distinct groups of gram-negative bacteria, and they are also characteristic for lipid A.<sup>48, 69</sup>

In summary, despite the variations discussed, lipid A when compared to the other lipopolysaccharide regions exhibits rather little structural variability.

### Synthetic Lipid A

Based on the results of chemical analysis, lipid A has been chemically synthesized.<sup>40</sup> The first fully synthetic lipid A molecule (preparation 506 or LA-15-PP) corresponds in structure to *E. coli* lipid A (see Fig. 4A). Later, other lipid A's and lipid A partial structures were prepared that all contain a  $\alpha$ (1-6)-linked D-glucosamine disaccharide but that differ in the acylation and phosphorylation pattern. These preparations include the heptacyl species of *S. minnesota* lipid A (Fig. 4B, compound 516 or LA-16-PP), the tetraacyl precursor Ia (406 or LA-14-PP), the pentacyl precursor Ib (LA-20-PP), an isomer of precursor Ib (LA-21-PP), as well as the 1-dephospho (e.g., compound 504 or LA-15-PH) and the 4'-dephospho (505 or LA-15-PF) partial structures of *E. coli* lipid A and acyl-deficient compounds such as precursor Ia. Also, lipid A disaccharide analogues such as the pentacyl compound 316, with an acylation pattern distinct from that of bacterial lipid A, have been chemically synthesized.<sup>35, 40</sup> Further, a great number of monosaccharide partial structures with a different acylation and phosphorylation pattern have been prepared by several groups.<sup>1, 33, 35, 52</sup> These compounds include synthetic counterparts of lipid A-related bacterial products such as lipid X and lipid Y, as well as other partial structures and analogues corresponding to either the reducing or the distal hexosamine unit of the lipid A backbone.

### PHYSICAL STRUCTURE OF ENDOTOXIN

Lipopolysaccharides and free lipid A are amphiphilic molecules. In aqueous solution, therefore, and above the critical micellar concentration, they are not present as individual molecules but rather as aggregates that may adopt different three-dimensional (or supramolecular) structures. Among the supramolecular arrangements that may be assumed by amphiphiles are micellar, hexagonal, lamellar, and nonlamellar cubic as well as inverted hexagonal structures (Fig. 6).<sup>51</sup> The particular physical structure formed depends on intrinsic chemical parameters, i.e., the primary chemical structure, and extrinsic factors, i.e., ambient conditions such as temperature, pH, and concentration of divalent cations, in particular  $Mg^{2+}$  and

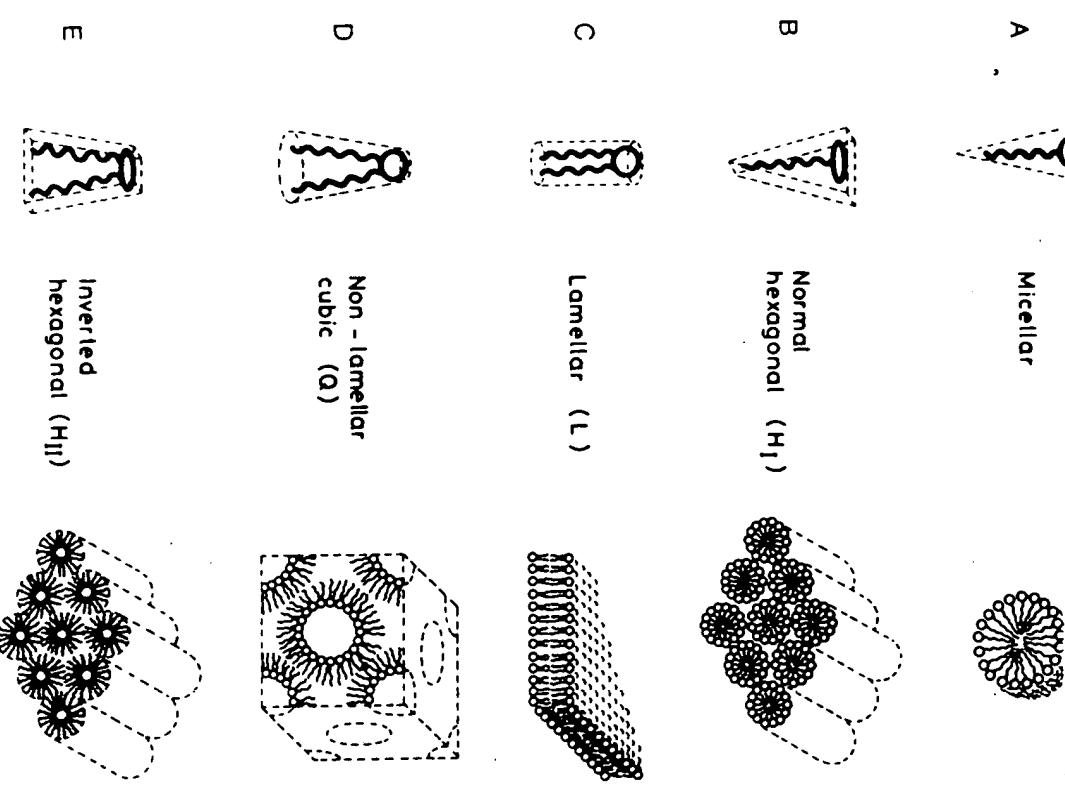


Figure 6. Schematic representation of molecular shape of amphiphiles and the corresponding supramolecular structure. (From Seydel U, Brandenburg K: Conformation of endotoxin and their relationship to biological activity. In Nowotny A, Spitzer JI, Ziegler E [eds]: Cellular and Molecular Aspects of Endotoxin Reactions [Endotoxin Research Series, vol. 1.1]. Amsterdam. Elsevier, 1990, p 63; with permission.)

$Ca^{2+}$ . On elevation of the temperature within one type of structure, transitions at a phase transition temperature  $T_c$  can take place from the gel ( $\beta$ ) to the liquid-crystalline ( $\alpha$ ) state, reflecting an increase of the fluidity of the amphiphiles' hydrophobic region, i.e., the acyl groups. Frequently the  $\beta \leftrightarrow \alpha$  phase transition is associated with a transition between different three-dimensional structures.

The importance of the physical state of endotoxin aggregates in relation

the biologic activity has been stressed previously,<sup>1-10</sup> with the idea that by a unique primary structure and thus by a particular three-dimensional conformation and phase state, we have applied Fourier-transform infrared spectroscopy, differential scanning calorimetry, and x-ray small-angle diffraction with synchrotron radiation to determine these physical parameters of bioactive lipid A. The results obtained so far can be summarized as the following.

#### Fluidity (Phase States)

In the *S. minnesota* lipopolysaccharide series, the values of  $T_c$  are lowest for deep rough (Re) mutant preparations (around 30°C) and increase with the length of the polysaccharide portion S form lipopolysaccharides expressing a  $T_c$  around 37°C to 40°C. Free lipid A possesses the highest  $T_c$  value (approximately 45°C). Adding bivalent cations or lowering the pH causes a significant rigidification of the acyl chains of free lipid A and lipopolysaccharide preparations and also leads to an increase in  $T_c$ . Under alkaline conditions (pH > 7), a fluidization of the lipopolysaccharide and A acyl chains and a decrease in  $T_c$  takes place. Free lipid A and all investigated rough mutant lipopolysaccharides exhibit an extremely strong lyotropic behavior, i.e., a strong dependence of the  $\beta \leftrightarrow \alpha$  chain-melting transition on the water content. Thus, for example, the lipid A phase transition is clearly observed only at water concentrations higher than 50% to 60%.<sup>9</sup>

#### Three-dimensional Structure

For free lipid A of *S. minnesota* and *E. coli* we have established complete phase diagrams, i.e., the dependence of the structural polymorphism on temperature, water content, and  $Mg^{2+}$  concentrations were established.<sup>8, 76, 77</sup> Their main features are the following:

1. The  $\beta \leftrightarrow \alpha$  acyl chain-melting transition is—under all conditions—connected with a change in the supramolecular structure of the lipid assembly. Within the transition range, usually different phases coexist.
2. In the gel ( $\beta$ ) state, cubic phases (Q) are predominant at high water content (>60%) and at high [lipid A]:[ $Mg^{2+}$ ] ratios. In the low water-concentration range, the lamellar phase (L) is the exclusive structure at all  $Mg^{2+}$  concentrations.

3. In the liquid crystalline ( $\alpha$ ) state, the hexagonal  $H_h$  phase is predominant. Its contribution is weak, however, at low  $Mg^{2+}$  concentration and high water content but becomes significant at higher  $Mg^{2+}$  concentration and lower water content.

4. Under near-physiologic conditions of water content, divalent cation concentration, and temperature, the lipid A assemblies adopt cubic structures almost exclusively.

A similarly complex polymorphism appears to exist for Re mutant lipopolysaccharide. Thus,

1. Under physiologic conditions of temperature, bivalent cation con-

centration, and water content, Re mutant lipopolysaccharide aggregates preferentially assume nonlamellar (Q) structures.<sup>50</sup>

2. Inverted  $H_h$ -structures are observed only at high, physiologically nonrelevant temperatures (>70°C), but the transition from cubic to  $H_h$ -structures is decoupled from the  $\beta \leftrightarrow \alpha$  chain-melting transition.

For both Re mutant lipopolysaccharide and free lipid A, the  $\beta \leftrightarrow \alpha$  chain-melting transition as well as the transitions between different structures is reversible with temperature, at least after longer incubation.

In other studies applying small-angle x-ray or neutron scattering, only lamellar structures were found for free lipid A. The pentaacyl lipid A analogue 316, which carries two 14:0(3-OH) residues in position 2 and 2' and 14:0 groups in position 3,4 and 6', was found to have its acyl chains in an  $\alpha$  (liquid)-type arrangement and to adopt a nonlamellar inverted ( $H_h$ ) supramolecular structure.<sup>41</sup> The monosaccharidic lipid A precursor lipid X, on the other hand, appears to aggregate in micellar structures with its two acyl groups (14:0(3-OH)) being very mobile and in the  $\alpha$ -phase.<sup>44</sup>

## STRUCTURE-ACTIVITY RELATIONSHIPS

The successful elucidation of the chemical (and, to a certain extent, physical) structure of segments of the endotoxin molecule as well as the chemical synthesis of corresponding (partial) structures has provided a theoretical and experimental basis to recognize bioactive regions and to establish relationships between structure and activity at a molecular level. In the following, aspects of the relation of primary and tertiary structure to biologic activity of lipopolysaccharides will be discussed in the field of immunoreactivity, endotoxicity in vivo, and mediator induction in vitro.

#### Reactivity with Antibodies (Epitope Specificity)

**O-Specific Chain.** During the last decades it has been shown that the O-specific chain is involved in many of the biologic activities of endotoxins.<sup>50, 52, 53, 65</sup> Perhaps most significantly, the O-specific chain determines the serologic specificity of lipopolysaccharides and the bacteria containing them. The O-immunogenic and O-antigenic properties of lipopolysaccharide are determined by so-called O-factors, the chemical structure of which has been elucidated, in many cases, and correlations established between the structural and serologic features of a defined lipopolysaccharide or its parental bacterial strain. As an example, Figure 2A shows the structure of the repeating unit of the O-specific chain of *S. abortus equi* lipopolysaccharide in which abeequose (Abe) determines O-factor 4 while the  $\alpha$ -D-Glc(1-4)-D-Gal disaccharide, together with the backbone trisaccharide, determines O-factor 12 specificity. In *S. typhi* (see Fig. 2B) the same repeating unit (with factor 12) is present except that Abe is positionally replaced by tyvelose (Tvv), which determines a different specificity (O-factor 9). It is obvious that Abe and Tvv differ from each other chemically only in the orientation of two hydroxyl groups (positions 2 and 4), and this small structural alteration results in distinct serologic specificities.

inhibitors of harmful endotoxin effects. As a rule, however, they neutralize only that particular lipopolysaccharide against which they have been engendered. This serotype specificity also extends to the impressive protective activity of O-specific antisera in gram-negative bacterial infection models.<sup>7,4</sup>

**Inner Core.** The Kdo-containing inner core, being a common structural element of lipopolysaccharide, has been considered an attractive target for antibodies that might cross react with lipopolysaccharide of different bacterial origin and that may possibly provide crossprotection against pathogenic gram-negative bacteria and their endotoxins.<sup>11, 53, 59</sup> We have defined the epitope specificity of four monoclonal antibodies<sup>1, 6, 22, 23</sup> engendered against enterobacterial Re mutant bacteria, the lipopolysaccharide of which consists of an  $\alpha(2\text{-}4)$ -linked Kdo disaccharide and lipid A (Fig. 7). In these studies, the use of chemically synthesized homogeneous Kdo-containing antigens proved to be essential. Two of these antibodies (clones 17 and 22) were found to bind to the  $\alpha(2\text{-}4)$ -Kdo disaccharide and parts of the lipid A region, clone 17 specificity not being required, but clone 22 requiring the presence of phosphoryl groups in lipid A (see Fig. 7). It is noteworthy that the majority of antibodies present in polyclonal antisera raised against Re mutant bacteria were of the same specificity as clones 17 and 22. These monoclonal and polyclonal antibodies were identified as binding specifically to Re mutant lipopolysaccharides and as not crossreacting with other R- or S-form lipopolysaccharides. In contrast to clones 17 and 22 as well as to the polyclonal anti-Re specificities—which all represent anti-LPS antibodies—two other clones (20 and 25) were characterized to be true anti-core antibodies. Thus, clone 25 antibody was shown to react specifically with an  $\alpha(2\text{-}4)$ -linked Kdo disaccharide, which is expressed by *S. minnesota* Re (and Rb<sub>2</sub> mutant) lipopolysaccharide (see Fig. 7), among others. Clone 20, on the other hand, recognizes an  $\alpha$ -pyranosidically linked Kdo monosaccharide residue that is present in a terminal or lateral position in various lipopolysaccharides, and indeed, this antibody cross reacts with a series of lipopolysaccharides. Recent studies indicate that the clone 20 antibody binds also to wild-type bacteria and that it exhibits protective activity in certain experimental infection models.<sup>51</sup>

The specificity of these lipid A antibodies is independent of the acylation pattern, i.e., fatty acids are not part of the epitopes. Acyl groups may, however, modulate the exposure of lipid A determinants.<sup>5</sup> This was recognized when synthetic lipid A antigens containing the same hydrophilic backbone structure (1,4'-bisphosphorylated  $\beta(1\text{-}6)$ -linked D-glucosamine disaccharide) but differing in the number, type, and distribution of fatty acids were tested as native or liposome-incorporated antigens in the passive hemolysis inhibition assay for their reactivity with anti-*E. coli* lipid A antiserum.

The inhibition values for various native antigens varied from 2 to 500 ng, e.g., preparation hexaacyl *E. coli* lipid A (compound 506) exhibiting low and heptaacyl *S. minnesota* (compound 516) high inhibition values. The inhibitory capacity of these antigens, however, was comparable and significantly better (0.5–1 ng) if the preparations were tested after incorporation into liposome membranes. This could mean that under physiologic conditions, notably those preparations which in aqueous solution show high inhibition values (i.e., poor inhibition as compound 516), adopt preferentially nonlamellar structures rendering the immunoreactive sites, which are located in the lipid A backbone, less accessible to recognition by antibody. In the liposomal membrane, however, the lipid A preparations occur in a lamellar arrangement in which the lipid A backbone is exposed, allowing an optimal expression of its epitopes. Phosphate groups, on the other hand, are essential in lipid A epitope expression, but it cannot be decided at present whether they are part of the determinants or whether they generate an immunoreactive conformational state of lipid A.

Anti-*E. coli* lipid A antibodies cross react with a large variety of free lipid A's of distinct bacterial origin, i.e., those containing lipid A's harboring a bisphosphorylated hexosamine disaccharide. It is emphasized, however, that these lipid A antibodies do not cross react, at least *in vitro*, with lipopolysaccharide, i.e., with the lipid A component carrying the saccharide portion. Thus, in lipopolysaccharide the described lipid A epitopes (Fig. 8) are either not expressed (e.g., free lipid A may represent a neoantigen,

*Figure 7.* Schematic chemical and antigenic structure of the lipopolysaccharide of the *E. coli* Re mutant strain F515.<sup>4, 6, 22, 59</sup> Shown are the epitopes recognized by four monoclonal antibodies (clones 17, 20, 22, and 25). The clone 22 antibody recognizes, like clone 17, the glycosyl backbone of Re lipopolysaccharide; it requires, however, in addition, the 4'- and possibly the glycosidic-phosphoryl group. Acyl = fatty acid residues.



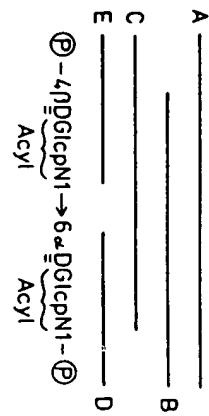


Figure 8. Schematic diagram of the epitope specificities of anti-lipid A antibodies. Shown is the lipid A backbone structure and disaccharide (A, B, C) and monosaccharide (D, E) reactive specificities. Data from references 4 and 5.

exposing determinants not present in lipopolysaccharide) or cryptic (e.g., Kdo or other lipopolysaccharide constituents may sterically hinder the binding of lipid A antibodies). There are, however, reports in the literature of mouse and human monoclonal antibodies of the IgM class that seem to be directed against lipid A and that cross react with different lipopolysaccharide.<sup>3, 88</sup> Their reactivity was elucidated in the ELISA assay system and it is known that ELISA determinations, unless certain precautions are taken, may lead to false results simulating cross-reactivity.<sup>16</sup> These antibodies exhibit protective activity in septicemic states,<sup>21, 95</sup> but further studies are required to understand their mechanism of action<sup>2, 95</sup> and to unequivocally define the epitopes they recognize.

#### Endotoxicity

Lipid A represents the lethal, leukopenic, pyrogenic—the endotoxic—principle of lipopolysaccharides. This association finally became clear after the demonstration that chemically synthesized *E. coli* lipid A (compound 506) expressed, with similar doses, the same spectrum of endotoxic effects as bacterial (*E. coli*)-free lipid A and endotoxin.<sup>22</sup> Thus, the endotoxic properties of lipopolysaccharide are essentially embedded in a molecule with the structure shown in Figure 4A.

*E. coli* lipid A possesses a complex structure, and it was not known which of its constituents was essential for or contributed to bioactivity. To determine this, various lipid A partial structures were prepared by either defined chemical degradation of bacterial lipid A or by total synthesis, and then they were analyzed *in vivo* for endotoxic activity. Such studies performed by our group and other groups emphasized the fever-producing capacity, and the results can be summarized as follows.<sup>14, 37, 65, 80</sup>

**1. Disaccharides with Two Phosphates.** As compared to hexaacyl *E. coli* lipid A, preparations having seven (compound 516) or five (compounds LA-20-PP and LA-21-PP) fatty acids exhibited reduced pyrogenicity; compounds with four acyl groups (406) were significantly less active, and compounds with only two fatty acids (preparation 606) were nonpyrogenic. Thus, the number of acyl chains present greatly influences bioactivity. A synthetic analogue (compound 316) containing five fatty acids but in an arrangement different than that seen in *E. coli* lipid A exhibits only marginal endotoxicity, showing that the location of acyl groups is also important for lipid A endotoxic activity. More detailed analyses regarding the contribution of the chain length of the fatty acids, the stereochemistry of hydroxylated acyl groups, and their nature (saturated versus unsaturated, 3-hydroxy

versus 3-oxo) have, because of the lack of corresponding synthetic structures, not yet been performed. Such factors might be important as indicated by the finding that pentaacyl lipid A of *Rhodobacter sphaeroides* harboring, like *E. coli*, a bisphosphorylated glucosamine disaccharide backbone, but unlike *E. coli*, containing short-chain ester-linked 3-hydroxy capric acid, an unsaturated fatty acid, and 3-oxo myristic acid, is endotoxically nonactive.<sup>39, 85</sup>

**2. Disaccharide with One Phosphate.** The 1- and 4'-monophosphoryl partial structures such as compounds 504 (LA-15-PH) and 505 (LA-15-HP) were, in general, less active in vivo than the bisphosphorylated parent compounds (here 506, LA-15-PP) as pyrogens or lethal toxins and in preparing for or in eliciting the local Shwartzman reaction. The lipid A component of *B. fragilis* lacks the nonglycosidic phosphate<sup>43, 82</sup> and has, in fact, very little endotoxic activity.

**3. Monosaccharides.** Hexosamine monosaccharide partial structures being phosphorylated at position 4 and acylated at various sites show low activity in some *in vitro* systems.<sup>1, 30, 82</sup> They lack, however, significant endotoxic *in vivo* activity in nonsensitized animals. Compounds with a phosphate group in position 1 and corresponding to the reducing moiety of lipid A (such as lipid X) are completely devoid of endotoxicity but possess antagonistic properties (see the following section). A monosaccharide bacterial correlate is represented by lipid A of *R. viridis*,<sup>71</sup> which exhibits no endotoxic activity *in vivo*.<sup>33</sup>

Collectively, these data suggest that full endotoxic activity is expressed by a molecule containing two hexosamine residues (*D*-gluco-configurated), two phosphoryl groups, and six fatty acids (saturated and, in part, 3-hydroxylated) including 3-acetoxyacyl groups in a defined location as they occur in *E. coli* lipid A. Partial structures lacking only one of these constituents, irrespective of its chemical nature; molecules containing different, although chemically related constituents; and analogues with a different arrangement of constituents are either less endotoxic or inactive. A unique molecular structure thus seems to allow optimal expression of endotoxicity *in vivo*.

#### Reactivity with Immune Cells (Mediator Induction In Vitro)

When entering the circulation of higher organisms, endotoxins interact with a number of humoral and cellular components of the host. Thus, lipopolysaccharide has been shown to bind to proteins such as high- and low-density lipoproteins, serum albumin, complement components, a 28 KDa factor, and perhaps most importantly to an acute-phase protein called lipopolysaccharide-binding protein (LBP).<sup>75, 84</sup> Some of these proteins probably prevent the random attachment of the amphiphilic lipopolysaccharide molecule to cells and tissues. They seem rather to mediate a more specific transport of lipopolysaccharide to organs involved in endotoxin accumulation, i.e., the liver and, to some extent, the spleen and lung. At these body sites and in the circulation, lipopolysaccharides interact with cells of the mononuclear phagocyte system, and this event is believed to play a decisive role in the mechanism of endotoxin action. Notably, monocytes and

macrophages have been identified as primary endotoxin target cells and have been shown to form and release a variety of mediator molecules that are endowed with distinct and potent biologic activities. According to present knowledge, the principal endogenous mediators include tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), interleukin 1 (IL-1), and interleukin 6 (IL-6). These secondary hormonelike mediators can induce many of the typical endotoxin effects such as fever by acting independently, in sequence, or synergistically.<sup>89</sup>

We hypothesized that the structure-activity relationships revealed in the *in vivo* system pyrogenicity may also be recognized in *in vitro* systems involving the production and release of those bioactive peptide mediators involved in producing fever. Therefore, we studied the structural prerequisites of endotoxins with respect to their capacity to induce IL-1 and TNF production in human peripheral monocytes.<sup>14,48,49</sup> In these studies, various *S.* and R-form lipopolysaccharides, lipid A's, and lipid A partial structures (bacterial and synthetic) were employed. Some of the TNF results obtained in numerous dose-response experiments with cells of different low- and high-responder donors are shown in Figure 9.

S- and notably R-form lipopolysaccharides were very potent TNF-inducers (concentration range, 1 pg to 1 ng/ml) as was synthetic *E. coli* lipid A (preparation 506), which, however, exhibited lower activity (concentration range 1 to 100 ng). Synthetic *S. minnesota* heptaacyl lipid A (preparation 516) and the monophosphoryl partial structures 504 and 505 were

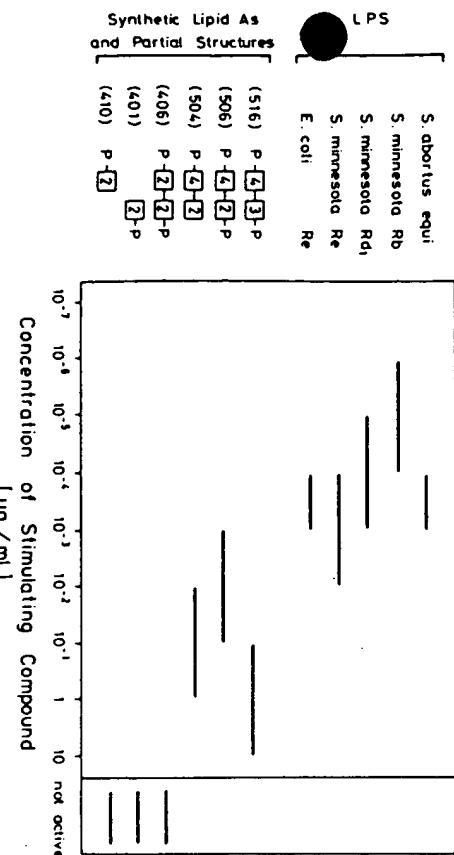


Figure 9. Tumor necrosis factor  $\alpha$  (TNF- $\alpha$ )-inducing capacity of lipopolysaccharides, lipoids, A, and partial structures. Shown is the minimal concentration of compounds required for the induction of less than 1 unit/ml of released TNF- $\alpha$  and the compounds used for stimulation of cells in a schematic representation. The boxes represent GlcN residues and the numbers in the schematic formulas indicate the number of fatty acids bound to either GlcN I or GlcN II. (From Loppnow et al: IL-1 induction capacity of defined lipopolysaccharide partial structures. *Immunol* 142:3229-3238, 1989; with permission.)

were significantly less active (concentration range 10 ng to 10  $\mu$ g). The tetracyl precursor Ia (compound 406), the bisacyl compound 606 (data not shown), and the monosaccharide structures 401 (lipid X) and 410 were completely inactive. The same hierarchy of lipopolysaccharide and partial structures was established for their IL-1-inducing capacity in human peripheral monocytes.<sup>46, 47</sup> Again, lipopolysaccharide was a very potent inducer of IL-1 release; free lipid A was less active whereas lipid A partial structures showed greatly reduced activity or—like lipid X (compound 401) and precursor Ia (compound 406)—no activity. On stimulation of cells with lipid X and precursor Ia, no intracellular IL-1 could be detected either.

In summary, the rules established for structure-activity relationships in these *in vitro* analyses parallel those deduced from *in vivo* assay systems such as pyrogenicity. In addition, it is noteworthy that R-form lipopolysaccharide was more active than free lipid A in inducing the release of TNF and IL-1. It therefore seems that the Kdo-containing inner core can upregulate lipid A bioactivity.<sup>28, 49</sup>

Antagonism of Mediator Induction In Vitro

Recently we have extended these studies by investigating whether inactive compounds such as precursor 1a (406) or lipid X (401) can antagonize lipopolysaccharide or lipid A-induced IL-1 or TNF production.<sup>38, 47, 50</sup> It was found that indeed tetracyl compound 406, added to cells either 1 hour before or simultaneously with lipopolysaccharide, completely inhibited lipopolysaccharide- (but not *Staphylococcus epidermidis*-) induced mediator production by human peripheral monocytes. We and others have further shown that the suppressive effect of compound 406 is related to an inhibition of the formation of TNF-specific or IL-1-specific mRNA. Endotoxin-induced TNF and IL-1 formation is also inhibited by the nontoxic lipopolysaccharides of *Rhodobacter sphaeroides*<sup>55</sup> and *Rhodobacter capsulatus*.

We are presently investigating the structural prerequisites that determine the capacity of compounds to antagonize endotoxin effects. As discussed previously, endotoxic (agonistic) activity of compounds containing the bisphosphorylated hexosamine disaccharide strongly depends on the number of fatty acids present. Thus, hexaacyl *E. coli* lipid A (compound 506) is a strong agonist (TNF and IL-1 induction), pentaacyl precursor II (1,4,20-PP) is less potent, and the tetracyl precursor Ia (406) is completely inactive. The last compound, on the other hand, has potent antagonistic properties whereas a compound with only two fatty acids (compound 606) is a weaker antagonist. Compound 406 with four fatty acids has thus lost endotoxicity but exhibits strong antagonistic activity. This could be established for human mononuclear cells. In the murine system, however, the tetracyl compound 406 expresses, as compared to hexaacyl 506, reduced but still significant endotoxicity.<sup>20</sup> On the other hand, lipopolysaccharide of *R. capsulatus*, also harboring only four fatty acids, is endotoxicologically nonactive in the mouse (and human) system and exhibits strong antagonistic activity. Tetracyl compound 406 and tetracyl *R. capsulatus* lipid A differ in the nature of their fatty acids: Compound 406 contains four 14:0(3-OH) residues whereas *R. capsulatus* harbors two amide-bound fatty acids with 14 carbons atoms (in the form of 3-oxo) but, unlike compound 406, the two ester

linked acyl residues have only 10 carbon atoms [10:0(3-OH)]. The different activity of these tetracyl compounds in the murine system may therefore depend on either their different natures or on the different chain lengths of the fatty acids present.

Compounds with three acyl groups [14:0(3-OH)] have also been investigated.<sup>52</sup> In their backbone structure these compounds correspond to the synthetic preparation 405 (precursor Ia lacking the 4'-phosphoryl group). It was found that a compound carrying two acyl groups at GlcN II and one at GlcN I induced TNF release in mouse macrophages, whereas the preparation with the reverse distribution of the three acyl groups was inactive. In fact, this latter compound proved to be a potent antagonist in this system, suggesting that the bisacylated GlcN I region of lipid A is important for antagonistic activity. The corresponding monosaccharidic partial structure lipid X (compound 401), which is completely devoid of toxicity, shows significant antagonistic activity *in vitro*<sup>52</sup> and *in vivo*.<sup>61</sup>

We consider studies along these lines to be important because they may provide deeper insight into the relationship between endotoxins and the host. Thus, it is remarkable that on biodegradation of *E. coli* lipid A by macrophages and granulocytes, two fatty acids are removed by the enzyme 3'-cytotoxicacylhydrolase, yielding a structural counterpart of precursor Ia (preparation 406). This compound is endotoxicologically less active and represents a potent endotoxin antagonist. On enzymatic detoxification and lipid A, a molecule is thus formed that counteracts endotoxin bioactivity. On the other hand, studies with antagonistic endotoxin partial structures may also lead to new strategies to prevent or intervene with endotoxin-induced shock states. The antagonistic action of endotoxicologically nonactive partial structures, which is observed if the antagonist is given shortly before, simultaneously with, or shortly after lipopolysaccharide challenge, should not be confused with the induction of cellular refractoriness ("tolerance"), which is induced by endotoxicologically active preparations and the development of which requires several hours.<sup>25</sup>

## THE BIOACTIVE STRUCTURE OF LIPID A

As discussed, it appears that the endotoxic potency of lipid A is determined by a peculiar primary structure consisting of a  $\beta$ 1,6-linked *D*-hexosamine disaccharide, two phosphoryl, and six acyl groups with *Kdo* attached in defined locations. This unique primary structure is likely to determine a peculiar conformation and supramolecular structure. Expressing this conformation, lipid A is bioavailable and capable of interacting, perhaps selectively, with humoral factors or cellular and subcellular targets of the endotoxin-susceptible host. We presently favor the view that for the expression of endotoxic activity a particular supramolecular structure including at least partial melting of acyl chains at physiologic temperature is required. It seems reasonable to assume that a higher fluidity of the hydrocarbon chains of lipid A should favor the interaction with the host cell membrane, the fluidity of which is higher at the physiologic temperature than that of bound or free lipid A. This means that those biologic effects

provoked by a direct incorporation of the lipid A portion into the host cell lipid matrix should proceed at a higher rate. This concept is supported by the fact that the biologically less active heptaacyl *S. minnesota* compound 516 exhibits a relatively high phase transition temperature ( $T_c \sim 48^\circ\text{C}$ ) while biologically highly active preparations such as Re mutant lipopolysaccharide have lower phase transition temperatures ( $T_c \sim 30^\circ\text{C}$ ). It is not known, however, which physical structure of lipid A is involved in endotoxic activity. Based on the acylation pattern of the biologically less active compound 516 leading to an increase of the volume of the hydrophobic region (as compared to the biologically active hexaacyl lipid A of *E. coli*, and as suggested by our serologic studies<sup>53</sup>) it may be assumed that heptaacyl lipid A adopts, in aqueous solution, to a larger extent more complex physical structures such as inverted phases. Also, a pentaacyl lipid A analogue (compound 316) that is endotoxicologically inactive is present in inverted states.<sup>41</sup> On the other hand, preparation 406, which is also less active than lipid A, is found in a bilayered lamellar arrangement using dried preparations whereas the endotoxicologically inactive compound lipid X preferentially adopts a micellar state.<sup>44</sup> It therefore appears that neither of these physical structures alone is responsible for triggering the initial steps of endotoxic events. Bacterial and synthetic *E. coli* lipid A can, under physiologic conditions, adopt both lamellar and inverted structures,<sup>55, 66, 77</sup> and it is possible that this ability to express both these conformations in a reversible manner is related to its potent endotoxic activity.

## CONCLUSIONS

The significance of endotoxin in bacteria-induced inflammatory processes is now widely appreciated as are the mechanisms leading to endotoxic manifestations involving the production of endogenous monocyte-derived bioactive peptide and lipid mediators. Also, great progress has been made in our understanding of the molecular structure of endotoxin. Lipid A has been proved to represent the toxic center of endotoxin, its primary structure has been elucidated, it has been chemically synthesized, and the principal structural requirements for its biologic activities are known. As a result of these studies, the concept emerged that, as compared with other phospholipids, the constitution of lipid A is unique and that, because of its unique primary structure, lipid A adopts a peculiar conformation that is responsible for its characteristic (endotoxic) biologic activities. In fact, recent studies have revealed that the biologic activity depends on its three-dimensional arrangement as well as the fluidity of its acyl chains. It is not understood, however, how the physical structure is important in the expression of biologic activity of lipid A.

The initial and therefore central event that triggers the reaction cascade resulting in endotoxic effects is the interaction of lipid A with either a humoral factor (like LBP) and subsequently the membrane or its direct interaction with the membrane of mononuclear phagocytes resulting in the production and release of toxic mediators. It is possible that lipid A, by way of its hydrophobic region, intercalates into the lipid matrix of the

mammalian cell in an unspecific way.<sup>79</sup> More likely, a particular binding molecule on the target cell is able to specifically recognize lipid A or lipid A/protein complexes. The presence of specific lipid A-binding proteins on host cells such as the 80 kDa LPS-binding protein<sup>57</sup> and the macrophage surface protein CD 14<sup>58</sup> has been experimentally demonstrated. So far, however, it is not known whether these recognition proteins are functional receptor molecules, which after binding of lipid A deliver an activation signal, or whether further steps (such as internalization of lipid A into the cell or binding to a cytosolic receptor) are required to initiate a biologic response.

In view of the unique structure of bioactive lipid A and the demonstration of particular humoral and cellular lipid A-binding proteins, we presently favor the concept that a peculiar conformation, i.e., a three-dimensional structure, interacts with a defined cellular recognition molecule and that this interaction represents the key event in endotoxin action. To understand it at the molecular level, all efforts should be directed at the characterization of the conformation of lipid A, of the cellular-binding proteins, and of the interaction between them.

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